

APPLICATION  
FOR  
UNITED STATES LETTERS PATENT

TITLE: HEPATITIS B VIRUS TREATMENT

APPLICANT: LEE A. MIZZEN, HONGWEI LIU and MARVIN SIEGEL

CERTIFICATE OF MAILING BY EXPRESS MAIL

Express Mail Label No. EL445376375US

I hereby certify under 37 CFR §1.10 that this correspondence is being deposited with the United States Postal Service as Express Mail Post Office to Addressee with sufficient postage on the date indicated below and is addressed to the Commissioner for Patents, Washington, D.C. 20231.

February 5, 2002

Date of Deposit

Signature

Lisa G. Gray

Typed or Printed Name of Person Signing Certificate

## **HEPATITIS B VIRUS TREATMENT**

The present application claims the benefit of the filing date of U.S. Serial  
 5 Number 60/266,733 (February 5, 2001). The contents of U.S.S.N. 60/266,733 are incorporated  
 by reference in the present application in their entirety.

### **Field of the Invention**

The field of the invention is hepatitis B virus immunotherapeutics.

### **Background of the Invention**

Hepatitis B Virus (HBV) is a non-cytopathic DNA virus that infects humans and may  
 result in two clinical outcomes. In the majority of clinical infections in adults (90-95%), the  
 virus is cleared after several weeks or months, and the patient develops a lifelong immunity  
 15 against re-infection. In the remaining cases, however, the virus is not eliminated from the  
 tissues, and the patient remains chronically infected. The sequelae of chronic infection are  
 serious: such individuals are highly likely to develop scarring of the liver tissue (cirrhosis) and  
 may eventually develop hepatocellular carcinoma.

There is a prophylactic vaccine against HBV, and many developed countries have  
 20 implemented childhood vaccination programs to reduce the overall risk of infection.  
 Unfortunately, since the morbidity and mortality resulting from chronic HBV infection occurs  
 over a period of decades, the impact of vaccination will not be realized until well into the  
 future. Indeed, the annual incidence of HBV infection in adults is expected to decline by less  
 than 5% over the next eight years. By 2008, over 150,000 new infections will occur annually  
 25 in the United States alone and even more are expected in Europe and Japan. These individuals  
 will constitute a tremendous reservoir of virus, from which as many as 20,000 to 40,000  
 chronic infections will arise per year. Clearly, despite the availability of a vaccine, chronic  
 HBV infection will continue to be a serious health problem for many years to come.

Current therapies for chronic HBV include alpha interferon (IFN- $\alpha$ ) and lamivudine.  
 30 These therapies are judged by their abilities to reduce viral load and bring about  
 seroconversion or the loss of the HBe antigen, a marker of HBV replication and high-titre

viremia. IFN- $\alpha$  can eliminate HBe, but only in about one third of patients, those with low viral burdens. This treatment is costly and is associated with significant unpleasant side effects. Lamivudine is a small molecule anti-viral agent that is very well tolerated when administered orally. This compound is effective in reducing viral load in patients, but relatively few patients respond with loss of HBe, and discontinuation of therapy usually leads to increase in viral load. On the other hand, continued therapy can lead to selection for lamivudine resistant mutant variants. Combination therapy with IFN- $\alpha$  and lamivudine has not shown enhanced efficacy. Clearly, a successful immunotherapy to treat HBV infection is highly desirable.

### **Summary of the Invention**

The present invention features compositions that include a stress protein, or a portion thereof, and an HBV antigen. These compositions are discussed at length below. We note here that their components can be obtained from a variety of sources and their length and content can vary. For example, the stress protein can be one that is naturally expressed by any mammal (*e.g.* a human or non-human primate) or any other class of organisms that expresses stress proteins (*e.g.*, a bacterium or mycobacterium); the stress protein and/or the HBV antigen can be full length, truncated, or extended by the addition of one or more amino acid residues; and, in addition, the content of the stress protein or HBV antigen can vary (for example, a stress protein, or a portion thereof, and an HBV antigen can contain one or more amino acid substitutions). Any variation must still result, however, in a composition that can induce or enhance an immune response against HBV in a mammal. Preferably, the immune response is substantial enough that an HBV-infected patient experiences an improvement (objective or subjective) in a sign or symptom of the infection. Accordingly, an antigen encompasses full-length and naturally occurring antigens as well as fragments and other variants thereof that, when administered to a subject (*e.g.*, by the methods described herein), elicits an immune response to one or more epitopes present within the fragment or variant.

Similarly, in addition to full-length or naturally occurring stress proteins, the compositions of the invention can include fragments of stress proteins that are immunostimulatory (*i.e.*, fragments that facilitate an immune response to an antigen). The stress protein, or the fragment thereof, facilitates an immune response when the immune

response is greater, or in any way superior to, the immune response that typically occurs when the HBV antigen is administered alone.

The immune response can be either a humoral or a cell-mediated response. For example, an antigenic fragment can contain one or more HLA class I peptide antigens, as described herein. A cell-mediated immune response involves antigen specific cells of the immune system, such as cytotoxic T lymphocytes (CTLs) as well as, possibly, T helper lymphocytes (Th) and cells of the innate immune system, such as monocytes, macrophages, dendritic cells, natural killer cells and  $\gamma\delta$  T cells. One of ordinary skill in the art is well able to detect or otherwise evaluate an immune response, which is evident by, for example, the induction of cytotoxic T lymphocytes (see the Examples below), a cellular proliferative response, induction of cytokines, or a combination of these events.

In particular embodiments, the HBV antigen can be the HBV core antigen or a fragment or derivative thereof. Derivatives of the HBV antigen include variants of the HBV antigen, such as those containing one or more amino acid substitutions (*e.g.*, conservative amino acid substitutions). For example, a variant of an HBV antigen can contain 1-2, 2-5, 5-10, 10-25, or more, substituted amino acid residues. Alternatively, substitutions or other mutations, such as deletions or truncations, can constitute 1-2, 2-5, 5-10, or 10-25% of the sequence of a full-length HBV antigen. Like the antigenic portion of the composition, a variant of a stress protein can contain one or more amino acid substitutions (*e.g.*, conservative amino acid substitutions). For example, a variant of a stress protein can contain 1-2, 2-5, 5-10, 10-25, or more, conservative amino acid substitutions. Here again, substitutions or other mutations, such as deletions or truncations, can constitute 1-2, 2-5, 5-10, or 10-25% of the sequence of a full-length stress protein.

Various combinations of stress proteins and HBV antigens are also within the scope of the invention. For example, the compositions of the invention include those in which a full-length HBV antigen is associated with a full-length stress protein; an antigen that consists of a fragment or other variant of an HBV antigen is associated with a full-length stress protein; a full-length HBV antigen is associated with a fragment or other variant of a stress protein; and a fragment or other variant of an HBV antigen is associated with a fragment or other variant of a stress protein. Of course, as described herein, more than one of each of these components (*i.e.*, more than one HBV antigen and more than one stress protein) may be present, and each of the

components may be present in the form of a full-length protein or an immunologically active fragment or variant thereof.

Moreover, in any of the arrangements described herein, the HBV antigen and the stress protein can be associated in any manner. For example, the stress protein and the HBV antigen, can be present in the form of a fusion polypeptide (wherein the stress protein and the HBV antigen are covalently linked during translation of a fused open reading frame). Alternatively, a stress protein and an HBV antigen can be linked by chemical conjugation after each has been translated or synthesized individually. The components can also be non-covalently associated (in, for example, a mixture or a more ordered composition). The terms “polypeptide” and “protein” are used interchangeably to describe a chain of amino acid residues, except where it is clear from the context that a distinct meaning is intended.

While stress proteins are discussed further below, we note here that the stress protein can be a heat shock protein (Hsp). Further, the Hsp can be a mycobacterial Hsp, such as Hsp65 (e.g., Hsp65 of *Mycobacterium bovis*), or any member of an Hsp family of proteins from any species.

The compositions of the invention can be formulated for administration to a subject in a variety of ways and, optionally, contain an adjuvant. Additional optional components of the composition include pharmaceutically acceptable diluents, excipients, and carriers.

The invention also features methods of treating an HBV infection in a subject (e.g., a mammal, such as a human) by administering a composition of the invention to the subject infected with HBV and methods of preventing (or reducing the likelihood of) an HBV infection in a subject (e.g., a mammal, such as a human) by administering a composition of the invention to the subject before they have been infected with HBV.

The components of the composition need not be directly administered to the subject as polypeptides. Instead, a nucleic acid encoding the stress protein, the HBV antigen, or a fusion protein containing one or more of each can be administered, and the protein, antigen, or fusion protein will be expressed in the subject *in vivo*. The nucleic acid can be a part of a viral vector, for example, a part of a viral vector genome, or encapsulated in, e.g., liposomes. Alternatively, the nucleic acid can be delivered as a naked nucleic acid, such as plasmid DNA driven by regulatory sequences operable in eukaryotic or mammalian cells. Methods of administering nucleic acid molecules are well known in the art.

The invention further includes the use of compositions of the invention (*e.g.*, HBV-containing fusion proteins, the nucleic acid molecules that encode them, and pharmaceutical compositions containing them) in the manufacture of a medicament for the treatment of hepatitis B virus infection in accordance with the methods described herein.

Other features or advantages of the present invention will be apparent from the detailed description, the drawings, and the claims. All patent applications, patents, and publications cited herein are incorporated by reference in their entirety.

### **Brief Description of the Drawings**

Fig.1 is a DNA sequence encoding HBV (subtype adw) core antigen (HBc) (SEQ ID NO:1).

Fig. 2 is the amino acid sequence of HBV (subtype adw) core antigen (SEQ ID NO:2).

Fig. 3 is the DNA sequence of construct hisHepCorT(149/87S97F), which encodes a histidine-tagged, truncated HBV core antigen (amino acids 1-149; SEQ ID NO:3).

Fig. 4 is the amino acid sequence encoded by the DNA sequence of Fig. 3 (SEQ ID NO:4).

Fig. 5 is the DNA sequence of construct hisHepCor(97F)Hsp65, which encodes a histidine-tagged fusion protein that includes a full length HBV core antigen and an Hsp65 protein (SEQ ID NO:5).

Fig. 6 is the amino acid sequence encoded by the DNA sequence of Fig. 5 (SEQ ID NO:6).

Fig. 7 is the DNA sequence of construct hisHepCorT(149/87S97F)Hsp65, which encodes a histidine-tagged fusion protein that includes a truncated (amino acids 1-149) HBV core antigen fused to the Hsp65 protein (SEQ ID NO:7).

Fig. 8 is the amino acid sequence encoded by the DNA sequence of Fig. 7 (SEQ ID NO:8).

Fig. 9 is the DNA sequence of construct HepCorT(151/97F)Hsp65, which encodes a fusion protein that includes a truncated (amino acids 1-151) HBV core antigen fused to the Hsp65 protein (SEQ ID NO:9).

Fig. 10 is the amino acid sequence encoded by the DNA sequence of Fig. 9 (SEQ ID NO:10).

Fig. 11 is the DNA sequence of construct HepCor(97F)Hsp65, which encodes a fusion protein that includes the full length HBV core antigen fused to the Hsp65 protein (SEQ ID NO:11).

Fig. 12 is the amino acid sequence encoded by the DNA sequence of Fig. 11 (SEQ ID NO:12).

Fig. 13 is a graph depicting the CTL priming activity (% corrected lysis v. effector:target ratio) in C57BL/6 mice immunized with various immunogens (HepCorT(151/97F)Hsp65; HepCor(97F)Hsp65; HepCorT (151/97F); HepCor(97F); and hisHepCorT(149/87S97F)Hsp65). The resulting CTL lytic activity was assayed against EL4 cells pre-pulsed with a control peptide, MUT-1.52-59.Kb. Control mice were injected with placebo (buffer).

Fig. 14 is a graph depicting the CTL priming activity (% corrected lysis v. effector:target ratio) in C57BL/6 mice immunized with various immunogens (as in Fig. 13). The resulting CTL lytic activity was assayed against EL4 cells pre-pulsed with the HBc antigen-specific peptide, HBc.93-100.Kb. Control mice were injected with placebo (buffer).

Fig. 15 is a graph depicting the CTL priming activity (% corrected lysis v. effector:target ratio) in C57BL/6 mice immunized with various immunogens (as in Fig. 13). The resulting CTL lytic activity was assayed against EL4.HBc.1D7 cells, which express hepatitis B core antigen. Control mice were injected with placebo (buffer).

Fig. 16 is a graph depicting the CTL priming activity (IFN- $\gamma$  (pg/ml) v. effector:target ratio) in C57BL/6 mice immunized with various immunogens (as in Fig. 13). The ability of resultant CTLs to secrete gamma interferon (IFN- $\gamma$ ) was assayed against EL4 cells co-cultured with the HBc antigen-specific peptide, HBc.93-100.Kb. Control mice were injected with placebo (buffer).

Fig. 17 is a graph depicting the CTL priming activity (TNF- $\alpha$  (OD<sub>410</sub>) in C57BL/6 mice immunized with various immunogens (as in Fig. 13). The ability of generated CTLs to secrete tumor necrosis factor alpha (TNF- $\alpha$ ) was assayed against EL4 cells co-cultured with the HBc antigen-specific peptide, HBc.93-100.Kb. Control mice were injected with placebo (buffer).

### Detailed Description

The invention relates to HBV antigen-containing compositions that are useful in treating or preventing HBV infection. The content of the compositions can vary, as described herein, but the compositions comprise a stress protein, or a portion (*e.g.*, a fragment) or derivative thereof, and an HBV antigen. Various materials and procedures suitable for use in the methods of the invention are discussed below.

Because nucleic acid sequences encoding stress proteins and HBV proteins are known and available, nucleic acid constructs encoding them (alone or as a fusion construct) can be readily prepared using methods routinely practiced in the art. For examples of nucleic acids encoding a stress protein (an Hsp) optionally coupled to an antigen see WO 89/12455, WO 94/29459, WO 98/23735, WO 99/07860, and references cited therein. Fusion proteins can be produced not only by recombinant techniques but also by post-translational conjugation of a stress protein (*e.g.*, an Hsp) and an HBV antigen. Conjugation techniques are described, for example, in Hermanson (*Bioconjugate Techniques*, Academic Press, San Diego, CA, 1996) Lussow *et al.* (*Eur. J. Immun.* 21:2297-2302, 1991), and Barrios *et al.* (*Eur. J. Immun.* 22:1365-1372, 1992). Such methods of conjugation include the use of coupling agents such as glutaraldehyde, carbodiimides, and bisdiazobenzidine; the use of heterobifunctional crosslinkers such as *M*-Maleimidobenzoyl-*N*-hydroxysuccinimide ester; or the use of cysteine residues (those naturally present and/or those recombinantly inserted) in the stress protein and the antigen to facilitate intermolecular disulfide bond formation.

Any HBV antigen is suitable for inclusion in a fusion protein or composition of the invention. A preferred HBV antigen is the HBV core antigen or a fragment or derivative thereof. To facilitate testing, the HBV antigen can optionally be modified to include known mouse MHC-restricted CTL epitopes such as, for example, mouse H-2K<sup>b</sup>-restricted CTL epitopes. An example of such a modification is described in the Examples (for example, in the adw strain of HBV, residue 97 is isoleucine – replacing this with phenylalanine generates a mouse H-2K<sup>b</sup>-restricted CTL epitope). In addition, the antigen can be modified to include human HLA epitopes from more than one HBV subtype (*e.g.* adw, ayw, adr or ayr). For example, a single amino acid substitution from a threonine to a valine at position 91 of the HBV core antigen shown in Fig. 2 would duplicate the sequence of a known HLA-A11-restricted CTL epitope found in both the adw and adr HBV subtypes. Other derivatives of the HBV core antigen



include truncations. Such truncations would include, but are not limited to, truncations in which all or part of the C-terminal arginine-rich domain is removed (amino acids 150 to 185 of HBc). Suitable truncated HBc fragments include, but are not limited to, fragments consisting of only the first N-terminal 149 amino acids, or the first 151 N-terminal amino acids of HBc. In any event, a suitable fragment of the HBc antigen (or any suitable HBV antigen) would ideally include one or more B or T cell epitopes (or one or more B cell epitopes and one or more T cell epitopes), preferably one or more CTL epitopes. Additionally, the terminal cysteine of the HBV core antigen can be removed or replaced with a different amino acid. Other modifications to the amino acid sequence could be made. Another example is a substitution in an anchor residue of a known HLA-restricted CTL epitope to enhance the binding affinity of the peptide to the MHC Class I molecule. Although these modified HBV core antigens are suitable for inclusion in fusion proteins, they can also be used alone (optionally formulated with an adjuvant) to generate an immune response to HBV.

Additional HBV antigens suitable for use in the present invention include the HBV core antigen, HBV e antigen (HBeAg), x protein (HBx), polymerase polypeptide, and the HBV envelope proteins S, M, and L and fragments thereof (Seeger and Mason, *Microbiol. Mol. Biol. Rev.* 64: 51-68, 2000; Ganem and Schneider, *Hepadnaviridae: The viruses and their replication*. In: Knipe, DM and Howley, PM, eds. *Fields Virology*, Philadelphia: Lippincott Williams & Wilkins, 2001:2923-2969).

As described above, the HBV antigen, the stress protein, or both, can contain one or more amino acid substitutions (*e.g.*, conservative amino acid substitutions). These substitutions can be, but are not necessarily, made at one or more predicted non-essential amino acid residues. A "conservative amino acid substitution" is one in which one amino acid residue is replaced with another amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (*e.g.*, lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.*, glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (*e.g.*, threonine, valine, isoleucine) and aromatic side chains (*e.g.*, tyrosine, phenylalanine, tryptophan, histidine). Regardless of whether the substitution is designed to occur at a predicted non-essential site or is introduced randomly

along all or part of an HBV antigen or stress protein coding sequence (such as by saturation mutagenesis), the resultant mutants can be screened for antigenic and immunostimulatory activity, respectively, to identify mutants that retain biological activity. Following mutagenesis, the encoded protein can be expressed recombinantly and the activity of the protein can be determined.

The HBV antigen can be fused to either the N-terminus or C-terminus of the stress protein, with or without a linker or intervening exogenous sequence. In alternative embodiments, two HBV antigens (which can be naturally occurring or variant, as described herein) can be attached to the stress protein (one at the N-terminus and the other at the C-terminus of the stress protein; both at the N-terminus; or both at the C-terminus). Additionally, one or more HBV antigens (again, naturally occurring or fragments or other variants thereof; from either the same or different HBV proteins) can be attached either to the N-terminus or C-terminus, or both, of the stress protein. Additional alternative arrangements can be made, and will be evident to one of ordinary skill in the art, if more than one stress protein is included.

A stress protein and an HBV antigen (or combinations thereof; for example a stress protein and two or more HBV antigens) can be linked by chemical conjugation after each has been translated or synthesized individually. As noted above, the components can also be non-covalently associated (in, for example, a mixture or a more ordered composition). Compositions containing stress proteins or immunostimulatory fragments thereof that are non-covalently associated with an HPV antigen can be produced as described in U.S. Patent Nos. 6,048,530; 6,017,544; 6,017,540; 6,007,821; 5,985,270; 5,948,646; 5,935,576; 5,837,251; 5,830,464; or 5,750,119. See also, U.S. Patent Nos. 5,997,873; 5,961,979; 6,030,618; 6,139,841; 6,156,302; 6,168,793; and International Publication No. WO 97/06821.

Moreover, more than one type of viral antigen can be included in the composition. For example, in addition to the HBV antigen, compositions of the invention can include (or encode; any proteins described herein may be administered directly or by way of nucleic acids) an antigen from a different pathogen. Thus, in addition to an HBV antigen, the compositions can include (or encode) a hepatitis C antigen, a herpes simplex virus (HSV) antigen, a human immunodeficiency virus (HIV) antigen, a cytomegalovirus (CMV) antigen, an Epstein-Barr virus (EBV) antigen, a respiratory syncytial virus (RSV) antigen, a human papillomavirus (HPV) antigen, a herpes virus antigen, or a combination thereof. The same alternatives that have been

described for the embodiments in which the compositions contain only HBV as the viral antigen (e.g., the method of association with the stress protein, the inclusion of full-length, fragmented, or variant proteins, the variable number of components, and their arrangement) are applicable to the embodiments in which at least one HBV antigen and at least one other viral antigen are present in (or encoded by) the composition.

Surprisingly, it has also been found that removing the C-terminal arginine-rich domain from the core antigen results in a polypeptide capable of eliciting an immune response to the core antigen, particularly a cellular and/or a CTL immune response. The arginine-rich domain of the core antigen is located between amino acids 150 to 183 of the core antigen (Nassal, *J. Virol.* 66: 4107-4116, 1992). Suitable core antigen fragments include, but are not limited to, those that lack all or part of this region. For example, suitable core antigen fragments may contain of the first 149 or 151 amino acids (or fewer than 149 or 151 amino acids).

The compositions of the invention can optionally include an adjuvant. Examples of adjuvants that may be effective include, but are not limited to: Freund's complete adjuvant (FCA), Freund's incomplete adjuvant (FIA), SAF, muramyl dipeptide (MDP), lipopolysaccharide (LPS), lipid A, monophosphoryl lipid A (MPL), pertusis toxin (PT), stearyl tyrosine,  $\gamma$  inulin, RIBI (which contains three components extracted from bacteria), Quil-A, saponins (QS21), alum (aluminum hydroxide, aluminum phosphate), calcium phosphate, MF-59, immunostimulatory complexes (ISCOMS), CpG oligonucleotides and cytokines (Gupta and Siber, *Vaccine* 13: 1263-1276, 1995; Singh and O'Hagan, *Nature Biotechnology* 17: 1075-1081, 1999).

A suitable fragment or derivative of an HBV antigen will ideally contain at least one B or T cell epitope (or both). In a preferred embodiment, the fragment or derivative will contain at least one CTL epitope.

A variety of stress proteins have been isolated, cloned, and characterized from a diverse array of organisms (Mizzen, *Biotherapy* 10:173-189, 1998). Any immunostimulatory Hsp or immunostimulatory fragment thereof is suitable for use in the fusion polypeptides and compositions. For example, Hsp70, Hsp60, Hsp20-30 (low molecular weight Hsp), and Hsp10 (the GroES homologue) are among the major determinants recognized by host immune responses to infection by *Mycobacterium tuberculosis* and *Mycobacterium leprae*. In addition, Hsp65 of Bacille Calmette Guerin (BCG), a strain of *Mycobacterium bovis*, was found to be an effective immunostimulatory agent, as described in the example below.

Families of stress genes and proteins for use in the present invention are well known in the art and include, for example, Hsp100-200, Hsp100, Hsp90, Lon, Hsp70, Hsp60, TF55, Hsp40, FKBP, cyclophilins, Hsp20-30, ClpP, GrpE, Hsp10, ubiquitin, calnexin, and protein disulfide isomerases. See, e.g., Macario, Cold Spring Harbor Laboratory Res. 25:59-70, 1995; Parsell *et al.*, *Rev. Genet.* 27:437-496, 1993; and U.S. Patent No. 5,232,833.

Examples of Hsp100-200 proteins include Grp170 (for glucose-regulated protein). Grp170 resides in the lumen of the ER and in the pre-Golgi compartment, and may play a role in immunoglobulin folding and assembly.

Examples of Hsp100 proteins include mammalian Hsp110, yeast Hsp104, and *E. coli* ClpA, ClpB, ClpC, ClpX and ClpY.

Examples of Hsp90 proteins include HspG in *E. coli*, Hsp83 and Hsc83 in yeast, and Hsp90alpha, Hsp90beta, and Grp94 (small gp96) in humans. Hsp90 binds groups of proteins that are typically cellular regulatory molecules, such as steroid hormone receptors (e.g., glucocorticoid, estrogen, progesterone, and testosterone receptors), transcription factors, and protein kinases that play a role in signal transduction mechanisms. Hsp90 proteins also participate in the formation of large, abundant protein complexes that include other stress proteins.

Lon is a tetrameric ATP-dependent protease that degrades non-native proteins in *E. coli*.

Examples of Hsp70 proteins include Hsp72 and Hsc73 from mammalian cells, DnaK from bacteria or mycobacteria such as *Mycobacterium leprae*, *Mycobacterium tuberculosis*, and *Mycobacterium bovis* (such as Bacille-Calmette Guerin; referred to herein as Hsp71), DnaK from *E. coli*, yeast, and other prokaryotes, and BiP and Grp78. Hsp70 is capable of specifically binding ATP as well as unfolded polypeptides and peptides, and participates in protein folding and unfolding as well as in the assembly and disassembly of protein complexes.

Examples of Hsp60 proteins include Hsp65 from mycobacteria. Bacterial Hsp60 is also commonly known as GroEL. Hsp60 forms large homooligomeric complexes, and appears to play a key role in protein folding. Hsp60 homologues are present in eukaryotic mitochondria and chloroplasts.

Examples of TF55 proteins include Tcp1, TRiC, and thermosome. The proteins typically occur in the cytoplasm of eukaryotes and some archaeobacteria, and form multi-membered rings, promoting protein folding. They are also weakly homologous to Hsp60.

Examples of Hsp40 proteins include DnaJ from prokaryotes such as *E. coli* and mycobacteria and HSJ1, HDJ1, and Hsp40. Hsp40 plays a role as a molecular chaperone in protein folding, thermotolerance and DNA replication, among other cellular activities.

FKBP examples include FKBP12, FKBP13, FKBP25, and FKBP59, Fpr1 and Nepl. The proteins typically have peptidyl-prolyl isomerase activity and interact with immunosuppressants such as FK506 and rapamycin. The proteins are typically found in the cytoplasm and the endoplasmic reticulum.

Cyclophilin examples include cyclophilins A, B, and C. The proteins have peptidyl-prolyl isomerase activity and interact with the immunosuppressant cyclosporin A.

Hsp20-30 is also referred to as small Hsp. Hsp20-30 is typically found in large homooligomeric complexes or possibly heterooligomeric complexes. An organism or cell type can express several different types of small Hsps. Hsp20-30 interacts with cytoskeletal structures and may play a regulatory role in the polymerization/depolymerization of actin. Hsp20-30 is rapidly phosphorylated upon stress or exposure of resting cells to growth factors. Hsp20-30 homologues include alpha-crystallin.

ClpP is an *E. coli* protease involved in degradation of abnormal proteins. Homologues of ClpP are found in chloroplasts. ClpP forms a heterooligomeric complex with ClpA.

GrpE is an *E. coli* protein of about 20 kDa that is involved in the rescue of stress-damaged proteins as well as the degradation of damaged proteins. GrpE plays a role in the regulation of stress gene expression in *E. coli*.

Hsp10 examples include GroES and Cpn10. Hsp10 is found in *E. coli* and in the mitochondria and chloroplasts of eukaryotic cells. Hsp10 forms a seven-membered ring that associates with Hsp60 oligomers. Hsp10 is also involved in protein folding.

Ubiquitin has been found to bind proteins in coordination with the proteolytic removal of the proteins by ATP-dependent cytosolic proteases.

In addition to full-length stress proteins, any immunostimulatory fragments or derivatives would be useful in the present invention. An immunostimulatory fragment or derivative (e.g., an immunostimulatory fragment of an Hsp) is a fragment or derivative that facilitates an immune response to an antigen. The fragment or derivative can facilitate an immune response in a number of ways. For example, the fragment can induce an immune response that would not otherwise occur or enhance an immune response that would. A number of immunostimulatory

fragments have been described. Suitable fragments include, but are not limited to fragments comprising: (a) amino acids 161-370 of mycobacterial Hsp70 (particularly *M. tuberculosis* Hsp70) (Huang *et al.*, *J. Exp. Med.* 191:403-408; 2000, US patent application 09/761,534 filed January 16, 2001); (b) the ATPase domain or peptide binding domain of mycobacterial Hsp70 (particularly *M. tuberculosis* Hsp70) (Young, US Serial No. 09/025,178 filed November 25, 1997); (c) amino acids 280-385 of murine Hsc70 (the constitutive member of the Hsp70 family) (Udono *et al.*, *Int. Immunol.* 13: 1233-1242, 2001); (d) amino acids 359-610 of *M. tuberculosis* Hsp70 (Wand *et al.*, *Immunity* 15: 971-983, 2001); (e) for (a) to (d), corresponding regions in Hsp70 homologs from other species, and (f) amino acids 1 to 200 of mycobacterial Hsp65 (particularly *M. bovis* Hsp65) (Chu *et al.*, US Serial No. 09/613,303 filed July 10, 2000).

The stress proteins useful in the present invention can be obtained from any suitable organism, including, but not limited to: Gram-positive bacteria, Gram-negative bacteria, enterobacteria (e.g., *E. coli*), mycobacteria (particularly *M. leprae*, *M. tuberculosis*, *M. vaccae*, *M. smegmatis*, and *M. bovis*), yeast, *Drosophila*, and vertebrates (e.g., avians such as chickens, or mammals such as rats, mice, or primates, including humans).

To make a therapeutic (e.g., an immunotherapeutic) composition containing a fusion polypeptide, the polypeptide can be recombinantly produced in bacteria, yeast, plants or plant cells, or animals or animal cells. For example, fusion polypeptides according to the invention can be produced by transformation (transfection, transduction, or infection) of a host cell with a fusion polypeptide-encoding DNA fragment in a suitable expression vehicle. Suitable expression vehicles include plasmids, viral particles, and phage. For insect cells, baculovirus expression vectors are suitable. The entire expression vehicle, or a part thereof, can be integrated into the host cell genome. In some circumstances, it is desirable to employ an inducible expression vector, e.g., the LACSWITCH<sup>®</sup> Inducible Expression System (Stratagene; La Jolla, CA).

Those skilled in the field of molecular biology will understand that any of a wide variety of expression systems can be used to provide the recombinant fusion polypeptide. The precise host cell and vector used is not critical to the invention.

Proteins and polypeptides can also be produced by plant cells. For plant cells, viral expression vectors (e.g., cauliflower mosaic virus and tobacco mosaic virus) and plasmid expression vectors (e.g., Ti plasmid) are suitable. Such cells and vectors are available from a

wide range of sources (e.g., the American Type Culture Collection, Manassas, VA; also, see, e.g., Ausubel *et al.*, *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, 1994). The methods of transformation or transfection and the choice of expression vehicle will depend on the host system selected. Transformation and transfection methods are described, e.g., in Ausubel *et al.*, *supra*. Expression vehicles may be chosen from those provided, e.g., in Pouwels *et al.*, *Cloning Vectors: A Laboratory Manual*, 1985, Supp. 1987. The host cells harboring the expression vehicle can be cultured in conventional nutrient media adapted as needed for activation or repression of a chosen gene, selection of transformants or amplification of a chosen gene.

Where appropriate or beneficial, the nucleic acid encoding a fusion polypeptide can include a signal sequence for excretion of the fusion polypeptide, e.g., to facilitate isolation of the polypeptide from a cell culture. Specific initiation signals may also be required for efficient translation of inserted nucleic acid sequences. These signals include the ATG initiation codon and adjacent sequences. In some cases, exogenous translational control signals, including, perhaps, the ATG initiation codon, must be provided. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression can be enhanced by the inclusion of appropriate transcription or translation enhancer elements (e.g., ones disclosed in Bittner *et al.*, *Methods in Enzymol.* 153:516, 1987). Additionally the gene sequence can be modified for optimal codon usage in the appropriate expression system, or alternatively, the expression host can be modified to express specific tRNA molecules to facilitate expression of the desired gene.

It would be useful if the fusion polypeptides were soluble under normal physiological conditions. Also within the invention are methods of using fusion proteins (or other configurations of proteins, including covalent and non-covalent complexes and mixtures) in which the stress protein (or an immunostimulatory fragment thereof) and the HBV antigen are fused to (or otherwise associated with) an unrelated third protein or polypeptide to create at least a tripartite protein or mixture of proteins. The third protein may facilitate purification, detection, or solubilization of the fusion or other complex, or it may provide some other function. For example, the expression vector pUR278 (Ruther *et al.*, *EMBO J.* 2:1791, 1983) can be used to

create lacZ fusion proteins. The pGEX vectors can be used to express foreign polypeptides as fusion proteins containing glutathione S-transferase (GST). In general, such fusion proteins are soluble and can be easily purified from lysed cells by adsorption to glutathione-agarose beads, followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned target gene product can be released from the GST moiety.

A fusion protein or covalent complex can be purified using an antibody that specifically binds a portion of the fusion or complex. Alternatively, other properties of the protein included can be exploited for purification (*e.g.* metal binding). For example, a system described in Janknecht *et al.* (*Proc. Natl. Acad. Sci. USA.* 88:8972, 1981) allows for the ready purification of non-denatured fusion proteins expressed in human cell lines. In this system, the gene of interest is subcloned into a vaccinia recombination plasmid such that the gene's open reading frame is translationally fused to an amino-terminal tag consisting of six histidine residues. Extracts from cells infected with recombinant vaccinia virus are loaded onto Ni<sup>2+</sup> nitriloacetic acid-agarose columns, and histidine-tagged proteins are selectively eluted with imidazole-containing buffers. The same procedure can be used for a bacterial culture.

Alternatively, the third protein can be an immunoglobulin Fc domain. Such a fusion protein can be readily purified using an affinity column.

Fusion polypeptides, particularly those containing short antigenic fragments, can also be produced by chemical synthesis (*e.g.*, by the methods described in *Solid Phase Peptide Synthesis*, 2nd ed., 1984 The Pierce Chemical Co., Rockford, IL).

Once isolated, the fusion polypeptide can, if desired, be further purified and/or concentrated, so long as further processing does not impair its ability to elicit (*e.g.*, by inducing or enhancing) an immune response sufficient for implementation of the methods of the invention. A variety of methods for purification and concentration are well known in the art (*see, e.g.*, Fisher, *Laboratory Techniques In Biochemistry And Molecular Biology*, Work and Burdon, eds., Elsevier, 1980), including ultracentrifugation and/or precipitation (*e.g.*, with ammonium sulfate), microfiltration (*e.g.*, via 0.45 µm cellulose acetate filters), ultrafiltration (*e.g.*, with the use of a sizing membrane and recirculation filtration), gel filtration (*e.g.*, columns filled with Sepharose CL-6B, CL-4B, CL-2B, 6B, 4B or 2B, Sephacryl S-400 or S-300, Superose 6 or Ultrogel A2, A4, or A6; all available



from Pharmacia Corp.), fast protein liquid chromatography (FPLC), and high performance liquid chromatography (HPLC).

The polypeptides within the compositions of the invention can include antigenic or immunostimulatory determinants, or the whole protein, of more than one stress protein and/or more than one HBV protein. Optionally, the peptides can include other sequences to which an immune response is desired.

The invention includes immunotherapeutic compositions containing at least one fusion polypeptide as described herein, and, optionally, a pharmaceutically acceptable carrier, such as a diluent, *e.g.*, saline, phosphate buffered saline, or a bicarbonate solution (*e.g.*, 0.24 M NaHCO<sub>3</sub>).

The carriers used in the composition are selected on the basis of the mode and route of administration, and standard pharmaceutical practice. Suitable pharmaceutical carriers and diluents, as well as pharmaceutical necessities for their use, are described in Remington's Pharmaceutical Sciences. An adjuvant, *e.g.*, a cholera toxin, *Escherichia coli* heat-labile enterotoxin (LT), liposome, or immune-stimulating complex (ISCOM), can also be included in the immunotherapeutic compositions.

The compositions can be formulated as a solution (suitable for intramuscular, intradermal, or intravenous administration), suspension, suppository, tablet, granules, a powder, a capsule, ointment, or cream. In preparing these compositions, one or more pharmaceutical carriers can be included. Examples of pharmaceutically acceptable carriers or other additives include solvents (*e.g.*, water or physiological saline), solubilizing agents (*e.g.*, ethanol, polysorbates, or Cremophor EL<sup>®</sup>), agents for rendering isotonicity, preservative, antioxidizing agents, excipients (*e.g.*, lactose, starch, crystalline cellulose, mannitol, maltose, trehalose, calcium hydrogen phosphate, light silicic acid anhydride, or calcium carbonate), binders (*e.g.*, starch, polyvinylpyrrolidone, hydroxypropyl cellulose, ethyl cellulose, carboxy methyl cellulose, or gum arabic), lubricant (*e.g.*, magnesium stearate, talc, or hardened oils), or stabilizers (*e.g.*, lactose, mannitol, maltose, polysorbates, macrogels, or polyoxyethylene-hardened castor oils). If necessary, glycerin, dimethylacetamide, sodium lactate, a surfactant, sodium hydroxide, ethylenediamine, ethanolamine, sodium bicarbonate, arginine, meglumine, or trisaminomethane is added. Biodegradable polymers such as poly-D,L-lactide-co-glycolide or polyglycolide can be used as a bulk matrix if slow release of the composition is desired (*see e.g.*, U.S. Patent Nos. 5,417,986, 4,675,381, and 4,450,150). As noted above, pharmaceutical preparations such

as solutions, tablets, granules or capsules can be formed with these components. If the composition is administered orally, flavorings and colors can be added.

The immunotherapeutic compositions can be administered via any appropriate route, *e.g.*, intravenously, intraarterially, topically, by injection (*e.g.* intraperitoneally, intrapleurally, subcutaneously, intramuscularly), orally, intradermally, sublingually, intraepidermally, intranasally (*e.g.*, by inhalation), intrapulmonarily, or rectally.

The amount of immunotherapeutic composition administered will depend, for example, on the particular stress protein/antigen composition, whether an adjuvant is co-administered with the composition, the type of adjuvant co-administered, the mode and frequency of administration, and the desired effect (*e.g.*, protection or treatment), as can be determined by one skilled in the art. In general, the immunotherapeutic compositions are administered in amounts ranging between 1  $\mu$ g and 100 mg per adult human dose. Preferably, between 50 to 10,000  $\mu$ g (*e.g.*, about 100 to 5000  $\mu$ g, especially about 500, 1000, 1500 or 2000  $\mu$ g) of the fusion protein is administered. If adjuvants are administered with the immunotherapeutic, amounts ranging between 1 ng and 100 mg per adult human dose can generally be used. Administration is repeated as necessary, as can be determined by one skilled in the art. For example, a priming dose can be followed by one or more booster doses at weekly or monthly intervals. A booster shot can be given at 3 to 12 weeks after the first immunization, and a second booster can be given at 3 to 12 weeks after the first booster, using the same formulation or a different formulation. Serum, PBLs, or PBMCs, can be taken from the individual for testing the immune response elicited by the immunotherapeutic against the HBV antigen included in the fusion protein. Methods of assaying antibodies or cytotoxic T cells or cytokine-secreting cells against a specific antigen are well known in the art. Additional boosters can be given as needed. By varying the amount of fusion polypeptide in the composition, the immunization protocol can be optimized for eliciting a maximal immune response.

Of course, the polypeptides (alone or as part of a fusion protein) can also be delivered by administering a nucleic acid, such as a viral vector (*e.g.*, a retroviral or adenoviral vector).

The immunotherapeutic of the invention can also be administered in combination with one or more compounds or compositions that have activity against HBV (an HBV antiviral). For example, a patient can first be treated with an HBV antiviral to reduce the severity of the HBV infection (as measured by, for example, reduction or loss of circulating HBe antigen (a marker of

HBV replication and high-titre viremia), appearance of anti-HBe antibodies, reduction or disappearance of serum HBV DNA or reduction in alanine aminotransferase (ALT) levels). Once a suitable reduction is achieved, the immunotherapeutic of the invention can then be administered to the patient. Alternatively, the HBV antiviral and the immunotherapeutic can be administered at substantially the same time (keeping in mind that the antiviral and the immunotherapeutic may have different routes of administration), or the immunotherapeutic can be administered first, followed by treatment with the antiviral. Antiviral compounds or compositions suitable for use in such combinations with the immunotherapeutic include, but are not limited to interferon- $\alpha$ 2b (Intron A, Schering Plough), pegylated interferon- $\alpha$ 2b, and nucleoside analogs such as lamivudine [(-)- $\beta$ -L-3'-thia-2',3'-dideoxycytidine or 3TC] (Epivir-HBV, Glaxo Wellcome) and ribavirin (Rebetron<sup>TM</sup>, ICN Pharmaceuticals). There are a number of additional experimental compounds which may be suitable, and these include: hemtricitabine (2', 3'-dideoxy-5'-fluoro-3'-thiacytidine, FTC, coviracil, Triangle Pharmaceuticals), clevudine (2'-fluoro-5-methyl- $\beta$ -L-arabinofuranosyl uracil, L-FMAU, Triangle), adefovir (9-(2-phosphonylmethyl)-adenine, PMEA, Gilead Sciences), entecavir (Bristol-Myers Squibb), (-)-beta-D-2, 6-diaminopurine dioxolane (DAPD),  $\beta$ -L-2', 3'-dideoxy-5-fluorocytidine ( $\beta$ -L-FddC),  $\beta$ -L-2', 3'-didehydro-dideoxy-5-fluorocytidine ( $\beta$ -L-Fd4C), and famciclovir.

Without further elaboration, it is believed that one skilled in the art can, based on the above disclosure and the example below, utilize the present invention to its fullest extent. The following example is to be construed as merely illustrative of how one skilled in the art can isolate and use the fusion polypeptides, and is not limitative of the remainder of the disclosure in any way. All publications cited in this disclosure are hereby incorporated by reference.

### **Examples**

#### **Example 1: Construction of HBV Core Antigen-Hsp Fusion Proteins**

General methods and procedures for constructing fusion proteins can be found in WO 94/29459, WO 98/23735, WO 99/07860, and references cited therein.

The gene encoding the HBV subtype adw was obtained from plasmid pBR/HBV (purchased from the ATCC, ATCC 45020). The coding sequence for the full length protein is shown in Figure 1, and the amino acid sequence of the full length protein is depicted in Fig. 2.

The gene encoding the full length *M. bovis BCG* Hsp65 protein was obtained from plasmid pET65 (see WO 99/07860).

Using these starting materials and appropriate primers, the following constructs were prepared and cloned into pET28a (Novagen) for protein production:

5           1.1    hisHepCorT(149/87S97F): DNA encoding a truncated HBV core antigen (amino acids 1 to 149). The construct also contains an N-terminal 20 amino acid sequence containing a histidine tag. This construct has two amino acid changes from the wild type protein: amino acid 87 was changed from asparagine to serine and amino acid 97 was changed from isoleucine to phenylalanine. These two changes were made to reproduce known mouse  
10 CTL epitopes. There is also an exogenous asparagine residue added to the C-terminus of the truncated protein. The DNA sequence is shown in Fig. 3, and the encoded amino acid sequence is shown in Fig. 4.

          1.2    hisHepCor(97F)Hsp65:. DNA encoding a fusion protein comprised of amino acids of the full length HBV core protein fused to the N-terminus of *M. bovis BCG* Hsp65.  
5 The construct also contains an N-terminal 20 amino acid sequence containing a histidine tag and has amino acid 97 changed from isoleucine to phenylalanine. There are two additional residues inserted between the HBV core protein and the Hsp65 protein: an asparagine and a valine. The DNA sequence is shown in Fig. 5, and the encoded amino acid sequence is shown in Fig. 6.

          1.3    hisHepCorT(149/87S97F)Hsp65: DNA encoding a fusion protein comprised of amino acids 1 to 149 of HBV core fused to the N-terminus of *M. bovis BCG* Hsp65. The construct also contains an N-terminal 20 amino acid histidine tag and has an additional asparagine residue between the HBV core protein and the Hsp65 protein. The DNA sequence is shown in Fig. 7, and the encoded amino acid sequence is shown in Fig. 8.

25           1.4    HepCorT(151/97F)Hsp65: DNA encoding a fusion protein comprised of amino acids 1 to 151 of the HBV core protein fused to the N-terminus of *M. bovis BCG* Hsp65 (with no extra amino acids inserted between the two sequences). The HBV core sequence was modified from the wild type sequence as follows: isoleucine 97 is changed to phenylalanine. The DNA sequence is shown in Fig. 9, and the encoded amino acid sequence is shown in  
30 Fig. 10.

1.5 HepCor(97F)Hsp65: DNA encoding a fusion protein comprised of the full length HBV core protein fused to the N-terminus of *M. bovis* BCG Hsp65 (with no extra amino acids inserted between the two sequences). Like the construct directly above, amino acid 97 of the HBV core was changed from isoleucine to phenylalanine. The DNA sequence is shown in Fig. 11, and the encoded amino acid sequence is shown in Fig. 12.

1.6 HepCorT(151/97F). DNA encoding a truncated HBV core antigen (amino acids 1 to 151). This construct, in addition to being truncated at amino acid 151, has one amino acid change from the wild type adw protein shown in Fig. 2: amino acid 97 was changed from isoleucine to phenylalanine in order to reproduce a known mouse CTL epitope.

1.7 HepCor(97F). DNA encoding the full length HBV core antigen. This construct has one amino acid change from the wild type adw protein shown in Fig. 2: amino acid 97 was changed from isoleucine to phenylalanine in order to reproduce a known mouse CTL epitope.

Additional constructs can be made using other stress proteins, such as Hsp70 from

*Mycobacterium tuberculosis*.

The HBc molecule was in some instances modified by introducing one or more amino acid substitutions into the HBc gene product in order to reproduce known mouse-specific CTL epitopes. One of the substitutions was introduced at amino acid position 87, where asparagine was replaced by serine. This substitution created the mouse CTL epitope (<sup>87</sup>SYVNTNMGL<sup>95</sup>) restricted by H-2K<sup>d</sup> (HBc.Kd) (Kuhrober, *et al.*, *Int. Immunol.* 9:1203-1212, 1997). The second substitution was introduced at amino acid position 97, where isoleucine was replaced by phenylalanine. This substitution created the murine CTL epitope (<sup>93</sup>MGLKFRQL<sup>100</sup>) restricted by H-2K<sup>b</sup> (HBc.Kb) (Kuhrober *et al.*, *J. Immunol.* 156:3687-3695, 1996). A DNA fragment encoding 20 amino acids that contains six histidine residues (His-Tag) was added to the N-terminus of some of the constructs to facilitate purification. The fusion proteins can easily be made without these modifications.

### Example 2: Protein Purification

The following abbreviations are used: BCG for *Mycobacterium bovis* var. Bacille-Calmette-Guérin; CV for column volume; ET for endotoxin; EU for endotoxin units; IB for

inclusion body or bodies; MT for *Mycobacterium tuberculosis*; and PBS for phosphate-buffered saline.

All constructs were grown in a 15 L fermentor (Braun ED). The bacterial cell-paste was stored at -70°C until used for protein purification.

## 2.1 Purification of hisHepCorT(149/87S97F)

*Cell Lysis:* Approximately 277 g of the frozen cell paste was mixed with 800 mL of Lysis Buffer (30 mM TRIS, 10 mM 2-mercaptoethanol, 2 mM EDTA, 0.2 mM PMSF, pH 8.5). Then, lysozyme was added to 200 µg/mL and 50 µL of Benzonase™. The cells were frozen overnight at -70°C, then thawed for one hour, aliquoted into 50 mL centrifugation tubes, stored on ice, and sonicated with a BRANSON Sonifier II fitted with a 0.5 inch tip at setting 9 for 6 times 45 seconds.

The cell debris and IB were separated from the supernatant by centrifugation at 17,000 RPM (Beckman, Avanti J-30, JA30.50 rotor) for 20 min at 4°C. The pellet was re-suspended in 25 mL per tube with Wash Buffer (30 mM TRIS, 10 mM 2-mercaptoethanol, 2% (v/v) Triton X-100, pH 8.5). After centrifugation at 22,000 RPM for 20 min at 4°C, the supernatant was discarded and 20 mL of 8 M urea, 30 mM TRIS, 10 mM 2-mercaptoethanol, 1 mM EDTA, 0.2 mM PMSF, pH 8.5 were added and incubated overnight at 4°C. The supernatant with the dissolved IB was harvested by centrifugation at 22,000 RPM for 30 min, then split into two parts and frozen at -70°C.

*Ni Chelating Chromatography:* 250 mL of Chelating Sepharose Fast Flow (Amersham-Pharmacia) was packed into a XK50/30 column (Amersham-Pharmacia). The resin was washed with 3 CV each of 50 mM EDTA, Milli-Q™ quality water, 0.5 M NaOH, 2 M NaCl, Milli-Q™ quality water, 70% (v/v) ethanol, and Milli-Q™ quality water. Then, the resin was charged with 200 mM of NiSO<sub>4</sub>, washed with Milli-Q™ quality water, and equilibrated with 5 CV of Start Buffer (6 M guanidine HCl, 30 mM TRIS, 2 mM 2-mercaptoethanol, 20 mM imidazole, pH 8.5).

One part of the sample was applied onto the column at 5 mL/min, then washed with Start Buffer at 10 mL/min until the monitored absorption at 280 nm reached a baseline. To remove ET, the column was washed with 5 CV of 6 M guanidine HCl, 30 mM TRIS, 2 mM 2-mercaptoethanol, 20 mM imidazole, 2% (v/v) Triton X-100, pH 8.5. Subsequently, the column

was washed with 6 M urea, 30 mM TRIS, 2 mM 2-mercaptoethanol, 20 mM imidazole, pH 8.5. then the protein was eluted with a 5 CV gradient from 0 to 500 mM imidazole in 6 M urea, 30 mM TRIS, 2 mM 2-mercaptoethanol, 20 mM imidazole, pH 8.5 at 5 mL/min.

This chromatographic step was repeated with the second part of the sample and the fractions containing relatively pure protein were pooled.

*Source 15Q Anion-Exchange Chromatography:* 60 mL of Source 15Q resin (Amersham-Pharmacia) was packed into a XK26/40 column (Amersham-Pharmacia). The resin was washed at 5 mL/min with 2 CV Milli-Q™ quality water, 3 CV 1 M NaOH, 3 CV Milli-Q™ quality water, 2 CV NaCl, 2 CV Milli-Q™ quality water, 2 CV of a mixture of 10% (v/v) acetic acid & 40% (v/v) *iso*-propanol, 2 CV Milli-Q™ quality water, then equilibrated with 3 CV Start Buffer (6 M urea, 30 mM TRIS, 10 mM 2-mercaptoethanol, 1 mM EDTA, pH 8.5).

The pooled sample from the previous step was applied to the column at 0.5 mL/min then washed with Start Buffer at 6 mL/min, until the monitored absorbance at 280 nm reached a baseline. The column was then washed with 10 CV of 2% (v/v) Triton X-100 in Start Buffer. The protein was eluted with 11 CV from 0 to 600 mM NaCl in Start Buffer.

This chromatographic step was repeated and the fractions containing relatively pure protein were pooled.

*Source 15S Cation-Exchange Chromatography:* 50 mL of Source 15S resin (Amersham-Pharmacia) was packed into a XK26/40 column (Amersham-Pharmacia). The resin was washed at 5 mL/min with 2 CV 1 M NaOH, 3 CV Milli-Q™ quality water, 2 CV NaCl, 2 CV Milli-Q™ quality water, 2 CV of a mixture of 10% (v/v) acetic acid and 40% (v/v) *iso*-propanol, 2 CV Milli-Q™ quality water, then equilibrated with 3 CV Start Buffer (6 M urea, 23 mM sodium acetate, 10 mM 2-mercaptoethanol, 1 mM EDTA, pH 4.8).

The pooled sample from the previous step was applied to the column at 3 mL/min, then washed with Start Buffer at 3 mL/min, until the monitored absorbance at 280 nm reached a baseline. The protein was partially eluted with 15 CV from 0 to 1,000 mM NaCl in Start Buffer at 6 mL/min, the reminder with 6 M guanidine HCl, 30 mM TRIS, 10 mM 2-mercaptoethanol, 1 mM EDTA, pH 8.5.

*Dialysis and Sample Formulation:* Subsequently, the pooled sample was dialysed against the following solutions, in the order given:

1. 4 L of 6 M urea, 30 mM TRIS HCl pH 8.5, 10 mM 2-mercaptoethanol, 1 mM EDTA, 0.8 M arginine, at 4°C, overnight.
2. 4 L of 3 M urea, 30 mM TRIS HCl pH 8.5, 2 mM 2-mercaptoethanol, 1 mM EDTA, 25% (w/v) sucrose, at 4°C, overnight.
3. 2 L of 30 mM TRIS HCl pH 8.5, 4.5 mM reduced glutathione, 0.5 mM oxidised glutathione, 0.8 M arginine, 25% (w/v) sucrose, at 4°C, overnight.
4. 2 L of 10 mM PBS pH 7.4, 4.5 mM reduced glutathione, 0.5 mM oxidised glutathione, 25% (w/v) sucrose, at 4°C, overnight.
5. Repetition of step 4.

Assuming each dialysis step was completed until equilibrium was reached, the final concentrations of the ingredients are: PBS 10 mM, reduced glutathione 4.5 mM, oxidized glutathione 0.5 mM, arginine  $\leq 1.48$  mM, urea  $\leq 0.25$  mM, sucrose 730 mM or 25% (w/v).

## 2.2 Purification of hisHepCorT(149/87S97F)Hsp65

*Cell Lysis:* 79 g of the frozen cell paste were mixed with 1,000 mL of Lysis Buffer (30 mM TRIS, 10 mM 2-mercaptoethanol, pH 7.5). The lysate was frozen overnight at -70°C. Then, it was thawed and lysozyme was added to 200  $\mu\text{g/mL}$  and the cells were incubated for one hour. A mixture of several proteinase inhibitors (40 mg/mL each of aprotinin, leupeptin, pepstatin) and 15  $\mu\text{L}$  Benzonase™ was added. The lysate was sonicated in a 250 mL Rosette Cooling Cell (Fisher) using a BRANSON Sonifier II fitted with a 0.5 inch tip at setting 7 for 6 times 60 seconds.

The cell debris and IB were separated from the supernatant by centrifugation at 23,000 RPM (Beckman, Avanti J-30, JA30.50 rotor) for 20 min at 4°C. Guanidine HCl was added to the supernatant to a concentration of 6 M, yielding 1,400 mL. The sample was divided into one 400 mL and two 500 mL fractions.

*Ni Chelating Chromatography:* 187 mL of Chelating Sepharose Fast Flow (Amersham-Pharmacia) was packed into a XK50/30 column (Amersham-Pharmacia). The resin, previously regenerated according to the recommendations of the manufacturer, was



equilibrated with 5 CV of Start Buffer (6 M guanidine HCl, 50 mM imidazole, 30 mM TRIS, 1 mM 2-mercaptoethanol, pH 7.5).

The 400 mL sample was applied onto the column at 10 mL/min, then washed with Start Buffer until the monitored absorption at 280 nm reached a baseline. To remove ET, the column was washed with 5 CV of 6 M guanidine HCl, 30 mM TRIS, 1 mM 2-mercaptoethanol, 2% (v/v) Triton X-100, pH 7.5. Subsequently, the column was washed with 8 M urea, 30 mM TRIS, pH 8.5. Then, the protein was eluted with a gradient from 0 to 500 mM imidazole in 8 M urea, 30 mM TRIS, 1 mM 2-mercaptoethanol, pH 7.5.

This chromatographic step was repeated using the two 500 mL fractions from the previous step. The fractions obtained in these three runs were pooled.

*Source 30Q Anion-Exchange Chromatography:* 167 mL of Source 15Q resin (Amersham-Pharmacia) was packed into a XK50/30 column (Amersham-Pharmacia). The resin was regenerated with 5 CV of 2 M NaCl, 1 M NaOH, Milli-Q™ quality water, 40% (v/v) iso-propanol, 10% (v/v) acetic acid, and Milli-Q™ quality water. Then, the column was equilibrated with 3 CV of Start Buffer (6 M urea, 30 mM TRIS, 10 mM 2-mercaptoethanol, pH 7.5).

The pooled fractions from the previous step were applied to the column at 10 mL/min, then washed with Start Buffer, until the monitored absorbances at 214 nm, 254 nm, and 280 nm reached a baseline. The protein was eluted with a gradient from 0 to 500 mM NaCl in Start Buffer at 6 mL/min. Fractions containing the desired protein were pooled.

*Ceramic Hydroxyapatite Chromatography:* 53 mL of Ceramic Hydroxyapatite was packed into a XK26/40 column (Amersham-Pharmacia), regenerated with 3 CV 1 M NaOH and 0.5 M sodium phosphate, pH 6.8. The column was then equilibrated with 6 M urea, 20 mM sodium phosphate, pH 6.8.

The pooled fractions from the previous column was applied at 5 mL/min, then washed with 6 M urea, 20 mM sodium phosphate, pH 6.8 until the monitored absorbances at 214 nm, 254 nm, and 280 nm reached a stable baseline. Impurities bound to the column while hisHepCorT(149/87S97F)Hsp65 was in the flow-through.

*Dialysis and Sample Formulation:* The flow-through from the previous chromatography was pooled (250 mL) and dialysed against the following solutions, in the order given:

1. 4 L of 3 M guanidine HCl, 10 mM sodium phosphate, 0.8 M arginine, 4.5 mM reduced glutathione, 0.5 mM oxidised glutathione, 25% (w/v) sucrose, at 4°C, overnight.
2. 4 L of 10 mM sodium phosphate, 4.5 mM reduced glutathione, 0.5 mM oxidised glutathione, 25% (w/v) sucrose, at 4°C, overnight.
3. Previous step was repeated

Assuming each dialysis step was completed until an equilibrium was reached, the final concentrations of the ingredients are: Sodium phosphate 10 mM; urea 1.85 mM; reduced glutathione 4.5 mM; oxidised glutathione 0.5 mM; sucrose 730 mM or 25% (w/v).

*Gel Filtration Chromatography:* A HiLoad 26/60 Superdex 200 (Amersham-Pharmacia) gel filtration column, pre-packed by the manufacturer, was regenerated with 1 M NaOH, then equilibrated with 10 mM sodium phosphate, 4.5 mM reduced glutathione, 0.5 mM oxidised glutathione, 25% (w/v) sucrose, pH 7.4.

The dialysed sample was split into three portions (30 mL, 20 mL, 20 mL) and individually run on the column in the equilibration buffer at 1.5 or 2 mL/min and the fractions containing the protein were pooled.

### 2.3 Purification of HepCorT(151/97F)Hsp65

*Cell Lysis:* 500g of frozen cell-paste was mixed with 2500mL of Lysis Buffer (30mM TRIS, 10mM 2-mercaptoethanol, 2mM EDTA, 0.1mM PMSF, 10mg/mL aprotinin, 10mg/mL leupetin, 5mM p-amino-benzamidine, 0.2mg/mL lysozyme, pH 7.5), then frozen at -70°C for a minimum of 2 hours.

The frozen cell suspension was thawed at 37°C, stored on ice and sonicated (Branson Sonifier 450, 3/4" tip) 4 times for 1 min. The lysate was centrifuged at 15,000g, the soluble fraction clarified at 64,000g, and the soluble sample retained. After adding 6M urea to the soluble fraction, it was divided into three equal sized portions.

*Source 30Q Chromatography:* A XK50/30 column (Amersham-Pharmacia) containing 190mL Source 30Q resin (Amersham-Pharmacia) was regenerated then equilibrated with 3 CV of Start Buffer (6M urea, 30mM TRIS, 10mM 2-mercaptoethanol, 1mM EDTA, pH 7.5).

One portion of the sample was applied to the column. The resin was washed with Start Buffer until the absorption at 280nm, 254nm, and 214nm reached a baseline, then the protein

was eluted in a 5 CV linear gradient from 0 to 500mM NaCl in Start Buffer. The fractions containing HepCor65T(151/97F) were pooled.

The chromatographic step was repeated with the other two portions of the sample. The pools of all three portions were combined and dialyzed against 6M urea, 25mM sodium acetate, 10mM 2-mercaptoethanol, 1mM EDTA, pH 5.5. The sample was then divided into two portions.

*Source 15S Chromatography pH 5.5:* A XK26/40 column (Amersham-Pharmacia) containing 50mL of Source 15S resin (Amersham-Pharmacia) was regenerated, then equilibrated with Start Buffer S1 (6M urea, 25mM sodium acetate, 10mM 2-mercaptoethanol, 1mM EDTA, pH 5.5).

One portion of the Source 30Q sample was applied onto the column, washed with a) Start Buffer S1 until the baseline was stable at 280nm, b) 10 CV of 2 %(v/v) Triton X-100 in Start Buffer S1, and c) with Start Buffer S1 until the 280nm baseline was stable. Finally the protein was eluted in a 18 CV linear gradient from 0 to 230mM NaCl. The remaining protein was stripped off the column with a 1M NaCl wash step.

The chromatographic step was repeated with the second portion from the Source 30Q. The fractions containing HepCor65T(151/97F) were pooled, adjusted to pH 4.8 with concentrated acetic acid, and divided into two portions.

*Source 15S Chromatography pH 4.8:* The Source 15S column was regenerated and equilibrated with Start Buffer S2 (6M urea, 25mM sodium acetate, 10mM 2-mercaptoethanol, 1mM EDTA, pH 4.8).

One portion of the Source 15S sample was applied onto the column, washed with a) Start Buffer S2 until the baseline was stable at 280nm, b) 10 CV of 2 %(v/v) Triton X-100 in Start Buffer S2, and c) with Start Buffer S2 until the 280nm baseline was stable.

The protein was eluted in a 10 CV linear gradient from 0 to 500mM NaCl in Start Buffer S2. The remaining protein was stripped off the column with a 2 CV 1M NaCl wash step and a final 3 CV 6M guanidine-HCl strip. The fractions containing HepCorT(151/97F)Hsp65 were pooled.

*Source 15S Chromatography pH 4.8 – Endotoxin Removal:* After dialyzing the pooled fractions from the previous step in Start Buffer S2, it was reapplied to the Source 15S column.

The Source 15S column was regenerated and equilibrated with Start Buffer S2. One half of the Source 15S sample was applied onto the column, washed with Start Buffer S2 until the baseline at 280nm was stable, then with 10 CV of 2% (v/v) Triton X-100 in Start Buffer S2, and again with Start Buffer S2 until the baseline at 280nm was stable.

5 The protein was eluted in a 4 CV 1M NaCl wash step and a final 3 CV 6M guanidine-HCl strip. The fractions containing HepCor65T(151/97F) were pooled and dialyzed in three steps into DPBS, 10% (w/v) sucrose.

#### 2.4 Purification of HepCor(97F)Hsp65

10 *Cell Lysis:* 200g of frozen cell-paste were mixed with 600mL of Lysis Buffer (30mM TRIS, 20mM 2-mercaptoethanol, 5mM EDTA, 0.1mM PMSF, 0.2mg/mL lysozyme, pH 7.5) and then stirred at 4°C for approximately 30 min.

The cell suspension was sonicated (Branson Sonifier 450, 3/4" tip, Setting 9) 4 times for 1 min. The lysate was centrifuged at 18,500g and the soluble sample retained. The protein solution was clarified by centrifugation for 20 min at 4°C at 108,850g.

5 *Ammonium Sulfate Precipitation:* To the clarified protein solution ammonium sulfate was added to 25% saturation and the protein pelleted at 10,000g. The pellet was re-suspended in Lysis Buffer.

20 *Acetic Acid Precipitation:* The protein solution was carefully adjusted to pH 4.5 with 1M acetic acid and then stirred for 20 min at 4°C. The protein was then pelleted for 10 min at 10,000g and 4°C. The protein pellet was re-suspended in Q Buffer A (6M urea, 30mM TRIS, 10mM 2-mercaptoethanol, 5mM EDTA, 0.1mM PMSF, pH 8.5).

25 *Q Sepharose High Performance Chromatography:* A XK50/30 column (Amersham-Pharmacia) containing 150mL Q Sepharose High Performance resin (Amersham-Pharmacia) was regenerated then equilibrated with 3 – 5 CV of Q Buffer A.

The sample was applied to the column and collected in the flow through. 2-mercaptoethanol was added to 150mM and the protein pool incubated for 1 hour at 4°C.

*Second Q Sepharose High Performance Chromatography:* The Q Sepharose High Performance column was regenerated then equilibrated with Q Buffer A.

30 The flow-through off the first Q Sepharose Fast Flow column was applied to the column and again collected in the flow through. 2-mercaptoethanol was added to 300mM.

Guanidine-hydrochloride was added to 6M. The protein sample was then incubated for 72h at RT, then filtered through A 0.22µM filter.

*Superdex 200 Gel Filtration Chromatography:* A XK50/90 column (Amersham-Pharmacia) containing 1800mL Superdex 200 resin (Amersham-Pharmacia) was equilibrated with 2 CV of GF Buffer (6M urea, 30mM TRIS, 20mM 2-mercaptoethanol, 2mM EDTA, pH 7.5).

The sample was divided into 10 equal portions of 70mL. Then the individual portions were processed on this column and the fractions containing HepCor(97F)Hsp65 pooled.

*Sephadex 25 Desalting Gel Filtration Chromatography:* A XK50/30 column (Amersham-Pharmacia) containing 300mL Sephadex 25 resin (Amersham-Pharmacia) was regenerated then equilibrated with GF25 Buffer (6M urea, 50mM acetic acid, 5mM NaOH, 1mM EDTA, 10mM 2-mercaptoethanol, pH 4.7).

The sample was divided into 75mL portions and subsequently processed. The protein containing fractions were pooled.

*SP Sepharose High Performance Chromatography:* 275mL SP Sepharose High Performance resin (Amersham-Pharmacia) were regenerated then equilibrated with SP Buffer A (6M urea, 50mM acetic acid, 5mM NaOH, 1mM EDTA, 10mM 2-mercaptoethanol, pH 4.7).

The pooled sample obtained in the previous step was mixed with the resin and incubated on a horizontal shaker for 30 min at RT. Then the slurry was packed into a XK50/30 column (Amersham-Pharmacia) and washed with 2 CV of SP Buffer A. The column was then washed with 15 CV of 2 %(v/v) Triton X-100 in SP Buffer A. The detergent was removed during a wash with 5 CV SP Buffer A and 2 CV of 1M NaCl in SP Buffer A. The protein was then eluted isocratically in 6M urea, 10 mM TRIS, pH 7.5.

*Copper Chelating Sepharose Fast Flow Chromatography:* A XK50/30 column (Amersham-Pharmacia) containing 180mL Chelating Sepharose Fast Flow resin (Amersham-Pharmacia) was regenerated, loaded with copper sulfate, then equilibrated with 2 CV Cu Buffer A (6M guanidine-hydrochloride, 30mM sodium phosphate, pH 7.0).

The sample was applied to the column, washed with 3 CV of Cu Buffer A, then with 5 CV of 2% (v/v) TritonX-100 in Cu Buffer A, finally with 3 CV of Cu Buffer A to remove the detergent. The protein was eluted with 300mM imidazole in Cu Buffer A. The protein was subsequently dialyzed in 5 steps into DPBS.

## 2.5 Purification of HepCorT(151/97F)

*Cell Lysis:* 425g of frozen cell-paste were mixed with 2.5L of ice-cold 10mM EDTA, 100mM NaCl, 1mM 2-mercaptoethanol, pH 8.0. After mixing the cell suspension, the cells were pelleted by centrifugation for 10 min at 10,500g. The cells were re-suspended in 50mM NaCl, 1mM EDTA, 1mM 2-mercaptoethanol, 0.2g/mL lysozyme, pH 8.0, mixed and incubated on ice for 1 hour.

The cell suspension was sonicated (Branson Sonifier 450, 3/4" tip, Setting 8) 2 times for 2 min. The lysate was centrifuged at 18,500g and the soluble sample retained.

*Ammonium Sulfate Precipitation:* To the soluble fraction ammonium sulfate was added to 25% saturation, then impurities were pelleted at 10,000g for 40min. To the supernatant further ammonium sulfate was added to 35% saturation. After mixing for 30 min the protein is pelleted by centrifugation at 10,000g for 30 min. The pellet was re-suspended in 1mM EDTA, 1mM 2-mercaptoethanol, pH 8.0 and clarified at 76,500g for 20 min.

*Second Ammonium Sulfate Precipitation:* The sample from the first precipitation was dissolved in 1mM EDTA, 1mM 2-mercaptoethanol, pH 8.0 and reprocessed by the same procedure as described above.

*Phenyl Sepharose Fast Flow Chromatography:* A XK50/30 column (Amersham-Pharmacia) containing 200mL Phenyl Sepharose Fast Flow resin (Amersham-Pharmacia) was regenerated, then equilibrated with 0.85M ammonium sulfate, 20 mM sodium phosphate, 1mM EDTA, 1mM 2-mercaptoethanol, pH 6.8.

To the sample, 1M sodium phosphate, 1mM EDTA, 1mM BME, pH 6.8 was added up to 10mM sodium phosphate and ammonium sulfate to 20% saturation.

One half of the sample was applied to the column and was washed with equilibration buffer until the absorption at 280nm reached a baseline. The protein was eluted with 300mL of a linear negative gradient to 20mM sodium phosphate, 1mM EDTA, 1mM 2-mercaptoethanol, pH 6.8. The fractions containing HepCorT(151/97F) were pooled.

*Second Phenyl Sepharose Fast Flow Chromatography:* The column was regenerated and equilibrated with 0.85M ammonium sulfate, 20 mM sodium phosphate, 1mM EDTA, 1mM 2-mercaptoethanol, pH 6.8.

The sample pool off the first Phenyl Sepharose FF column was diluted to 2.5 mg/mL protein with equilibration buffer. The diluted sample was then applied to the column, washed with equilibration buffer until the baseline at 280nm was stable and eluted with 300mL linear gradient to 20mM sodium phosphate, 1mM EDTA, 1mM 2-mercaptoethanol, pH 6.8.

5 The fractions containing HepCorT(151/97F) were pooled and the protein pelleted by addition of ammonium sulfate to 35% saturation and subsequent centrifugation at 12,000g for 50 min. The pellet was then re-dissolved in 700mL of 8M urea, 10mM sodium acetate, 30mM acetic acid, 25mM NaCl, 0.5mM EDTA, 5mM 2-mercaptoethanol, pH 8.0.

10 *SP Sepharose fast Flow Chromatography:* A XK50/30 column (Amersham-Pharmacia) containing 180mL of SP Sepharose Fast Flow resin (Amersham-Pharmacia) was regenerated, then equilibrated with 5CV of 8M urea, 10mM sodium acetate, 30mM acetic acid, 25mM NaCl, 0.5mM EDTA, 5mM 2-mercaptoethanol, pH 8.0.

5 One half of the sample was applied to the column and washed with equilibration buffer until the absorption at 280nm reached a baseline. The protein was eluted in a 600mL linear gradient from equilibration buffer to 10mM sodium acetate, 30mM acetic acid, 1M NaCl, 5mM 2-mercaptoethanol, 0.5mM EDTA. Finally, the column was stripped with 6M guanidine-HCl, 50mMTRIS, pH 8.5.

20 The procedure was repeated with the second half of the sample, then the fractions containing HepCorT were pooled and dialyzed against of 6M urea, 20mM TRIS, pH 8.5, 0.5mM EDTA, 5mM 2-mercaptoethanol, pH 8.5, finally against 6M urea, 20mM TRIS, 5mM 2-mercaptoethanol, pH 8.5.

*Source 30Q Chromatography:* A XK50/30 column (Amersham-Pharmacia) containing 150mL of Source 30Q resin (Amersham-Pharmacia) was regenerated, then equilibrated with 4 CV of Equilibration Buffer (6M urea, 20mM TRIS, 5mM 2-mercaptoethanol, pH 8.5).

25 One third of the sample was applied to the column, washed with 95% equilibration buffer and 5% Elution Buffer (6M urea 1mMNaCl, 20mM TRIS, 5mM 2-mercaptoethanol, pH 8.5), then the protein was eluted in a 1L linear gradient to 100% Elution Buffer.

30 The second and third part of the sample were processed accordingly. The fractions containing HepCorT were pooled, then dialyzed against 6M urea, 20mM sodium acetate, 20mM acetic acid, 0.5mM EDTA, 1mM 2-mercaptoethanol, pH 8.0.

*SP Sepharose High Performance Chromatography:* A XK50/30 column (Amersham-Pharmacia) containing 180mL of SP Sepharose High Performance resin (Amersham-Pharmacia) was regenerated then equilibrated with 5 CV of 6M urea, 20mM sodium acetate, 20mM acetic acid, 0.5mM EDTA, 1mM 2-mercaptoethanol, pH 8.0.

One half of the sample was applied to the column and washed with Equilibration Buffer until the absorption at 280nm reached a baseline.

Impurities were eluted in two linear gradients, i.e., from 0 to 1M NaCl in Equilibration Buffer, then in a linear gradient to Elution Buffer 2 (6M urea, 40mM sodium acetate, 10mM acetic acid, 0.5mM EDTA, 1mM 2-mercaptoethanol, pH 8.0). The column was washed with 2% (v/v) Triton X-100 in Elution Buffer 2, then with 10 CV Elution Buffer to remove the detergent. The protein was then eluted in a gradient to 6M urea, 50mM TRIS, 0.5mM EDTA, 5mM 2-mercaptoethanol, pH 8.0.

After repeating the procedure with the second half of the sample the fractions containing HepCorT(151/9F) were combined and the protein dialyzed in 2 steps into 5mM sodium phosphate, 50mM NaCl, 3.1mM urea, 20% (w/v) sucrose, pH 8.5.

## 2.6 Purification of HepCor(97F)

*Cell Lysis:* 100g of frozen cell-paste were mixed with 400mL of ice-cold 5mM EDTA, 5mM 2-mercaptoethanol, pH 8.0. After mixing the cell suspension, lysozyme was added to 0.2g/mL and the suspension mixed and incubated on ice for 1 hour.

The cell suspension was sonicated (Branson Sonifier 450, 3/4" tip, Setting 8) 2 times for 2 min, then 200mL of ice-cold 20mM sodium acetate, 5mM acetic acid, 3M ammonium sulfate were added and mixed, well. The suspension was sonicated (Branson Sonifier 450, 3/4" tip, Setting 8) 3 min. Finally, the lysate was centrifuged at 18,500g and the soluble sample retained.

*Ammonium Sulfate Precipitation:* The soluble fraction was diluted with 1L of 0.85M ammonium sulfate. 70g/L of solid ammonium sulfate were slowly added while mixing. After further 30 min of mixing, the suspension was centrifuged at 18,500g for 60 min. Afterwards, the pellet was re-suspended in 500mL 1mM EDTA, 5mM 2-mercaptoethanol. 113.4g/L ammonium sulfate were slowly added while mixing; the solution was mixed for another 30 min. The protein was pelleted at 76,500g for 20 min.



The pellet was re-dissolved by addition of 1M sodium phosphate, 1mM EDTA, 5mM 2-mercaptoethanol, 20% saturation ammonium sulfate to a final concentration of 5mM sodium phosphate.

*Phenyl Sepharose Fast Flow Chromatography:* A XK50/30 column (Amersham-Pharmacia) containing 200mL Phenyl Sepharose Fast Flow resin (Amersham-Pharmacia) was regenerated, then equilibrated with 0.85M ammonium sulfate, 5mM sodium phosphate, 1mM EDTA, 5mM 2-mercaptoethanol, pH 6.8.

The sample was applied to the column and was washed with equilibration buffer until the absorption at 280nm reached a baseline. The protein was eluted with a step-gradient to 6M urea. The fractions containing HepCorT were pooled and the protein precipitated by addition of ammonium sulfate to 32% saturation. After pelleting the protein at 12,100g for 50, it was re-dissolved in 500mL 8M urea, 5mM TRIS, 5mM 2-mercaptoethanol, pH 7.5.

*Source 30Q Chromatography:* A XK50/30 column (Amersham-Pharmacia) containing 150mL of Source 30Q resin (Amersham-Pharmacia) was regenerated, then equilibrated with 4 CV of Equilibration Buffer (6M urea, 5mM TRIS, 5mM 5-mercaptoethanol, pH 7.5).

The sample was applied to the column, washed with Equilibration Buffer. The protein was eluted in a linear gradient from Equilibration Buffer to 6M urea, 1M NaCl, 5mM TRIS, 5mM 2-mercaptoethanol, pH 7.5. The fractions containing HepCor(97F) were pooled and divided into three portions.

*SP Sepharose High Performance Chromatography:* A XK50/30 column (Amersham-Pharmacia) containing 190mL of SP Sepharose High Performance resin (Amersham-Pharmacia) was regenerated, then equilibrated with 5CV of 6M urea, 30mM sodium acetate, 10mM acetic acid, 1mM EDTA, 5mM 2-mercaptoethanol, pH 8.0.

One portion of the sample was applied to the column and washed with 6M urea, 40mM sodium acetate, 10mM acetic acid, 0.5mM EDTA, 1mM 2-mercaptoethanol, 2 %(v/v) Triton X-100, pH 8.0 for 20 CV. Then, in order to remove Triton X-100, the column was washed with 10CV of 6M urea, 40mM sodium acetate, 10mM acetic acid, 0.5mM EDTA, 1mM 2-mercaptoethanol, pH 8.0.

The protein was eluted in a 600mL linear gradient to 6M urea, 20mM TRIS, 1M NaCl, 1mM 2-mercaptoethanol, 0.5mM EDTA, pH 8.5.

The procedure was repeated with the other two portions of the sample, then the fractions containing HepCor(97F) were pooled. The protein was then dialyzed in 5 steps into 40mM sodium acetate, 0.05mM DTT, pH 6.5.

### 5 Example 3: Priming of mice for CTL activity

*Mice:* C57BL/6 (H-2<sup>b</sup>) mice were purchased from Charles River Laboratories (St. Constant, PQ).

*Cell lines:* The EL4 thymoma cell line (H-2<sup>b</sup>) was obtained from ATCC and cultured in Dulbecco's modified Eagles medium containing 10% FBS and 2 mM L-glutamine (DMEM-10). EL4.HBc.1D7 cells expressing HBc antigen were derived at Stressgen by transfecting 10 EL4 cells with a plasmid encoding the full length HBc gene and the neomycin resistance marker. The gene for the full-length HBc antigen was cloned from the adw subtype of HBV, and modified to encode known murine H-2Kb- and H-2Kd-restricted CTL epitope sequences (2 amino acid changes from the wild type protein adw protein were made: amino acid 87 was changed from asparagine to serine and amino acid 97 was changed from isoleucine to phenylalanine. These two changes were made to reproduce known mouse CTL epitopes.). Transfected cells were selected in DMEM-10 containing 1500 µg/mL G418 and cloned by limiting dilution to obtain the EL4.HBc.1D7 clone. Expression of HBc protein in this cell line was validated by Western immunoblot analysis using an HBc-specific antibody. The MHC Class I presentation of the H-2Kb-restricted CTL epitope was confirmed by lysis with a CTL line specific for this epitope. FACS analysis revealed a high level of MHC Class I expression on the transfectant, similar to that of the parental cell line.

*Priming of mice for CTL activity:* Mice were immunized (via subcutaneous injection in either the scruff of the neck or the interscapular region) with buffer or 2.9 nmol of one of the 25 following: HepCorT(151/97F)Hsp65, HepCorT(97F)Hsp65, hisHepCorT(149/87S97F)Hsp65, HepCorT(151/97F), or HepCorT(97F). At seven days following immunization, mice were euthanized by CO<sub>2</sub> inhalation or cervical dislocation and their spleens removed. Single cell suspensions of spleen cells were prepared in CTL medium (RPMI-1640, 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 µM 2-ME and 45 µg/mL gentamicin). 30 × 10<sup>6</sup> viable lymphoid cells were restimulated by incubating at 37°C / 5% CO<sub>2</sub> in the presence of 1 µM HBc CTL epitope peptide HBc.93-100.Kb, MGLKFRQL (Kuhober *et al.*, *J. Immunol.*

156:3687-95, 1996). This synthetic peptide (synthesized by Research Genetics, Huntsville, AL) used in the restimulation includes a murine CTL epitope restricted by H-2Kb.

The effector cells were harvested after 7 days and cultured in U-bottomed 96 well microtitre plates together with  $^{51}\text{Cr}$ -labelled target cells. Control EL4 targets were cells pre-pulsed with an irrelevant H-2Kb-restricted (MUT-1.52-59.Kb) peptide (see Fig. 13). Target cells were EL4 cells pre-pulsed with either HBc.93-100.Kb peptide (see Fig. 14) or with EL4.HBc.1D7 cells (see Figure 15). CTL (100  $\mu\text{l}$ ) were cultured with  $5 \times 10^3$  or  $1 \times 10^4$  target cells (100  $\mu\text{l}$ ) at various effector:target cell ratios (100:1, 33:1 or 11:1. To determine spontaneous release of label, an equal number of target cells were cultured without effector cells in a total of 200  $\mu\text{l}$  of CTL medium. Total release of label was determined by adding 100  $\mu\text{l}$  of Triton X-100 (2% v/v in water) to an equal number of target cells. After 4 hr incubation, the microtitre plates were centrifuged at  $200 \times g$  for 5 min and 100  $\mu\text{l}$  of culture supernatant were collected. The released radioactivity was determined by scintillation counting. The % corrected lysis was calculated according to the formula: % Corrected Lysis (CL) =  $100 \times (\text{CPM}_{\text{test}} - \text{CPM}_{\text{spont}}) / (\text{CPM}_{\text{total}} - \text{CPM}_{\text{spont}})$

*Cytokine analysis:* In order to quantitate the release of gamma interferon (IFN- $\gamma$ ) and tumour necrosis factor alpha (TNF- $\alpha$ ) from restimulated CTL, effector cells were seeded in U-bottomed 96 well microtitre plates and cultured together with 1  $\mu\text{M}$  HBc.93-100.Kb peptide and target cells at effector : target ratios of 100:1, 33:1 or 11:1. Supernatants were harvested after 4 or 24 hr incubation and analyzed for IFN- $\gamma$  (Figure 16) or TNF- $\alpha$  (Figure 17) levels by sandwich ELISA.